Potential of different fungi species in biodegradation field of phenolic compounds.

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Abstract

Phenolic compounds are dominant pollutants in terrestrial and freshwater environmental, that have toxic effects on living organisms at low concentrations, because it has the ability to persist in the ecosystem. So bio-removal is a good technique that employs the metabolic potential of microorganisms in order to clean up the environmental pollutants and turned into less dangerous or harmless substances. This work aims to the isolating of different species of fungi from wastewater of factories and coast of the red sea to test the ability of these fungi to degrade phenolic compounds. Ten species of fungi and sterile mycelium are used to remove phenol and its derivatives at different concentrations (0.4%, 0.6% and 0.8%). All fungi species have the ability of removing phenol and their derivatives, but *P.chrysogenum*, *Saccharomyces* sp. and sterile mycelium exhibited low ability to break down of hydroxyl-benzene, 2-naphthol and 1,3 dihydroxy benzene, respectively.

Key words: Biodegradation, phenolic compounds, fungi, chlorophyll pigment.

Introduction

Due to the release of phenolic compounds from agro-industrial operations, these compounds have become widespread in the world as environmental pollutants. Many of these aromatic compounds are toxic to the living system and their presence in the aquatic and terrestrial habitats often have serious ecological consequences, Where natural phenolic compounds are considered one of the most important and dangerous pollutants of the current environment [1]. Many industrial effluents and residues contain the structure of phenolic compound such as waste of ships, paper factories, aluminum factories, wine-distillery, olive oil extraction, green olive debittering, cork preparation, wood debarking, coffee production, coal gasification, coke-oven batteries, refinery and petrochemical plants and other industries that produce things such as synthetic chemicals, herbicides, pesticides, antioxidants, pulp-and-paper, photo developing chemicals, etc. [2-10].

These compounds are stable and even at low concentration they may be toxic towards living organisms and cause unfavorable chemical changes in water and soil

as inhibiting the sunlight penetration and decrease the photosynthetic activity of acquatic system [11]. Phenolic compound is the most toxic and it can persist in the ecosystem for long time due to its long range transportation, bioaccumulation in human and animal tissue and biomagnification in food chain [12]. Many serious diseases are caused by pollution phenol for both human and animal who inhalation and dermal contact such as cardiovascular diseases and severe skin damage, while ingestion can cause serious gastrointestinal damage and death. Even short-term application of phenol to the skin can produce blisters and burns in animals [13]. For these reasons several physico-chemical methods are used to remediate phenolic wastes such as ozonisation, adsorption, reverse osmosis, electrolytic oxidation, photocatalysis [14]. While all these methods have failing, so some of these methods are very costly like ozonisation, electrochemical, reverse osmosis and photochemical, but the disadvantage of physical adsorption is the elimination of sludge [15], bioremediation by using microbial cells to resolve phenol contamination problem consider one of the cheapest possible solutions [16-19].

The oxidative activities of microorganisms are the principal reason for the biological treatment of industrial wastewaters. Filamentous fungi may be an important supply of phenol degrading species [20]. Fungi are known for their wide incidence and also the outstanding capability of degrading advanced and inert natural products such as lignin, chitin and cellulose. Fungi adopt additional simply than bacterium and are capable to grow in extreme conditions, like nutrient deficiency, low pH, restricted water, etc. [21]. And not on the least, there comes the ability of fungi to survive within the presence of varied xenobiotics that turn to be toxic to variety of different microorganisms.

The purpose of this study was to investigate the ability of the different species of fungi to degrade some phenolic compounds, usually present in agro-industrial effluents and the effect of input and output degradation on chlorophyll pigments of *chlorella* sp.

Materials & Methods

Samples collection

Ten samples of wastewater were collected from different sites of red sea beach and factories in Upper Egypt during summer 2017. Wastewater samples were collected in sterile bottles (100 ml) and in plastic bags, respectively, transferred directly to the laboratory and preserved at 4°C until used.

Fungal isolation and identification

Fungi were isolated from the wastewater collected from red sea beach and factories in Upper Egypt using Czapek-Dox salts medium. water samples were suspended by vortexing and allowed to stand for several minutes. 1 ml portion was plated onto the Cz media containing 12 mg/mL tetracycline and streptomycin solution respectively in order to suppress the growth of bacterial colonies then incubated at room temperature for 7 days. The sample was subcultured three times to obtain a pure culture, which was transferred to a Cz slant and stored at 4 °C [22].The most common fungi were recultivated using Czapek's Dox medium until pure colonies were obtained. These fungi were identified by microscope using the methods described by [23].

Microorganisms

Eighteen isolates belonged to 6 genus and 10 species (*Alternaria alternate*, *Aspergillus flavus*, *A. fumgatius*, *A. niger*, *A. terreus*, *Cladosporium cladosporioides*, *penicillium aurantiogriseum*, *P. chrysogenum*, *Phoma* sp., *Saccharomyces* sp. and sterile mycelium) were isolated from wastewater and used for degradation of phenol and its derivatives.

Biodegradation media

Biodegradation was conducted on 4 combined media with 3 concentrations of phenol and their derivatives (0.4% , 0.6% and 0.8%), containing the following ingredients as single carbon and energy sources: hydroxy benzene, 2-naphthol, 4-nitrophenol and 1,3 dihydroxy benzene. The total concentration of phenolic compounds in each 100 ml were 0.4%,0.6% and 0.8%. Media contained also Chapek-Dox salts (in %), as follows: NaNO3 – 0.2, KH2PO4 – 0.1, KCI – 0.05, MgSO4.7H2O – 0.05, FeSO4.7H2O –0.001. The starting pH of culture media was 5.5 [24].

Determination of phenol degradation potential

The isolates showing growth on Chapek-Dox salts were used for further studies on bioremediation of phenol. Ten ml Chapek-Dox salts broths were inoculated with a 8-

d-old culture of the isolates, and the flasks were incubated under shake culture condition on a rotary shaker for 8 days at 28º C. After an appropriate incubation period, the cells were removed by centrifugation and the cell-free supernatants were used for estimation of residual phenol . The residual phenol was estimated by [25].

Folin –Ciocalteau reagent

The method employed the Folin –Ciocalteau phenol reagent (BDHL td) which was attended by the method of [26]. The general method involved the successive addition of 1.5 ml sodium carbonate (200 g^{-1}) and 0.5 ml Folin-Ciocalteau phenol reagent to 10 ml sample. After 60 min at 20º C, the absorbance was measured at 725 nm against distilled water and correct for the absorbance of a distilled water reagent blank.

$$\% \text{ Phenol Removal Efficiency (PRE)} = \frac{Ci - Cf}{Ci} \times 100$$

Wherever, Ci is the initial concentration of phenol (mg/L) and Cf is the final concentration of phenol. All experiments of phenol biodegradation were done three times; the results were expresses as average ± standard deviation (SD) [27].

Biological assay:

***Chorella* sp. test**

Each extract of *Aspergillus niger* and *Penicillium chrysogenum* (0.05 mg) was applied to 0.8 cm diameter filter paper disc (Whatman No.3), each disc was placed in test tube contained 10 ml of *Chorella* sp. The tubes were kept at a temperature of 25 ±1°C. A control tube with only *Chorella* sp. was also made, which incubated for 8 days.

Determination of pigments (chlorophyll a, chlorophyll b and carotenoids):

Pigment fractions were determined spectrocolorometrically according to [28]. A known volume (10 ml) of *Chorella* sp. suspension was centrifuged at 3000 rpm and the growth medium was decanted. Pigments were extracted in hot methanol (70ºC) for 10 minutes. Cell debris was removed by centrifugation and the clear supernatant, which contains the pigments was aspirated and diluted to a definite volume. The extinction coefficient was measured using spectrophotometer (Spectronic 601) against a blank of methanol at the wavelengths of 452, 644 and 663 nm. Taking into consideration the dilution made, the content of pigment fractions (µg/ml algal suspension) were calculated using the following equations [29]:

Chlorophyll *a* = 10.3 E_{663} - 0.918 E_{644}

Chlorophyll *b* = 19.7 E_{644}- 3.87 E_{663}

Carotenoids = 4.2 $E_{452.5}$ - [0.0264 Chl. *a* + 0.4260 Chl. *b*]

Statistical analysis

The experimental data were subjected to multivariate analysis of variance using anova. Means were compared using Duncan's test at the 5% level using the SPSS program (SPSS Inc., Chicago, IL, USA).

Results

Ten species and sterile mycelium belonging to 6 genera were collected in the present study (table, 1). *Aspergillus* was represented by 4 species, and *Penicillium* was displayed by 2 species, while the other genera were introduced by one species. *Saccharomyces* was the common genus in this studies, which comprising 46.68% of total fungi and recovered from 20% of the total samples. *Aspergillus* (*A. flavus, A. fumigatus*, *A. niger* and *A. terreus*) followed by *Saccharomyces* sp., which contributed by 16.67% of total fungi and isolated from 40% of total samples. *Penicillium* ranked the third place in the count, which comprising 13.33% of total fungi. The remaining species (*Alternaria alternata*, *Cladosporium cladosporioides*, *Phoma* sp. and sterile mycelium) were contributed collectively 23.33% of total fungi and isolated only from one sample.

Table (2) explained the Effect of different species of fungi on degradation of phenol derivatives at 0.4% concentrations, so the results showed that the ability of different fungi species on biodegradation of phenol derivatives were differed according to the type of phenol derivatives, so hydroxy-benzene and 1,3dihydroxy benzene exhibited the highest biodegradation by fungi species, but 2-Naphthol and 4-Nitrophenol showed the moderate bio removal by fungi species. The highest degradation of 1,3dihydroxy benzene was done by *Cladosporium cladosporioides* (90.40 %). While the lowest bio removal occurred by sterile mycelium for 1,3dihydroxy benzene (1.01%).

In general, table 3 showed that the ability of fungi species to degrade the phenol derivatives at 0.6% concentrations, so all species of fungi exhibited the degradation of phenol compounds by different proportions. Fungi species degrade phenol derivatives

191 in range between 1.1 to 87.08 %). *A. flavus* appeared highest ability for analysis of
192 1,3dihydroxy benzene compound (87.08), while *Saccharomyces* sp. showed lowest
193 degradation of 2-Naphthol compound (1.1%).

194 Table 4 . Appeared the potency of different species of fungi on degradation of phenol
195 compounds, where the ability of degradation of phenol and their derivatives increased
196 with increased the concentration of phenol based on the results detected in many
197 species of fungi. All species exhibited the potency to degrade phenol and their
198 derivatives, but the potency differed from species to species, and from derivatives to
199 derivatives. So *Cladosporium cladosporioides* showed the highest degradation of
200 hydroxyl benzene (95.00%), while sterile mycelium appeared the lowest degradation
201 of 1,3dihydroxy benzene (1.59 %).

202 In this study, we choose two species of degraded fungi of phenol and their
203 derivatives (*A.niger* and *P.chrysogenum*) at different concentration of phenol to find
204 out the ability of input and output of phenolic compounds degradation on
205 photosynthetic pigments of chlorella sp. (table, 5). Furthermore the value of
206 chlorophyll pigments was increased under output effects of phenolic compounds
207 degradation than input bioremoval. The results in table 5 explained that the highest
208 value of chlorophyll was showed at concentration 0.8% of 2-naphthol which degrade
209 by *P.chrysogenum* (12.83 mg/g fresh wt.), followed by concentration 0.8% of
210 hydroxyl-benzene which removal by *A.niger* (12.05 mg/g fresh wt.). While low value
211 of photosynthetic pigments of chlorella sp. was observed at concentration 0.4% for
212 control sample (3.75 mg/g fresh wt.).

213 # Discussion

214 The ability of microorganisms to eliminate injurious chemicals from contaminated
215 environments powerfully depends on the presence of different compounds. Most
216 industrial wastes embrace totally different organic mixtures creating vital the
217 investigation on the microbic destruction of composite substrates. The bioremoval or
218 degradation of one or all elements are often delayed and/or discontinued depending
219 on the composition of the studied mixture. Wastewaters from oil refineries, mining
220 business and variety of industrial chemical syntheses contain several aromatics as
221 phenol, cresols, nitrophenols, etc. [30]. The metabolism of aromatic compounds,

notably phenol and their derivatives explained in prokaryotic microorganisms[31,32].Ten species and sterile mycelium belonging to 6 genera were collected in the present study. *Aspergillus* was represented by 4 species , and *Penicillium* was displayed by 2 species, while the other genera were introduced by one species. *Aspergillus*, *Penicillium* and *Neurospora* attack aromatics and a variety of soil and wood-rotting fungi dissimilate the aromatic polymer lignin, as well as other plant phenolics [33]. Another fungus, the *Penicillium* strain Bi 7/2 has been shown the ability of growth on phenolic compounds as sole source of carbon and energy, including protocatechuic and gallic acids [34].

Aspergillus (*A. flavus, A. fumigatus*, *A. niger* and *A. terreus*) was followed *Saccharomyces* sp., which contributed by 16.67% of total fungi and isolated from 40% of total samples. Three species of fungi (*H. bergeri, F. oxysporum* and *A. flavus* var. *coulmnaris*) were the most common fungal species from the 25 samples of soils collected from the three Governorates (El Gharbia, Kafre El Sheikh and El-Menofia) [13].

From results in tables (2, 3 and 4), we have a tendency to showed that every one fungal species used have the ability to degrad the phenol and their derivatives. The microorganisms have the flexibility of removing phenol depended on the action of sort of enzymes. In bioremoval of phenol under aerobic conditions, the degradation is started by oxygenation in which the aromatic ring is initially monohydroxylated by a mono oxygenase phenol hydroxylase at a position ortho to the pre-existing radical to compose catechol. Catechol is that the main intermediate ensuing from metabolism of phenol by completely different microbic strains. Betting on the sort of strain, the catechol then undergoes a ring break down which will occur either at the ortho position so initiating the ortho pathway that results in the formation of succinyl Co-A and ethanoyl radical Co-A or at the meta position so initiating the meta pathway that results in the formation of pyruvate and acetaldehyde[1]. The results obtained from this investigation explained that, in generally the ability of different species of fungi for degradation of hydroxyl-benzene, 2-naphthol and 4-nitrophenol increased with increased the concentration of phenol and their derivatives. A lot of fungi species have ability to degrade phenol. Consequently, two species of fungi (*Mucor* sp. and *Rhizopus* sp.) have the ability with highest degardation of phenole at

initial concentration 100 mg/l. There is a relationship between the ability of fungi to analyze phenol with its concentration [27]. Many species of fungi produce extracellular enzymes for the metabolism complex carbohydrates into simple carbohydrates used by fungi as a source of sugar, for this reason, it has become possible to degrade pollutants such as phenol [35].

At the first three days, the ability of *Mucor* sp. and *Rhizopus* sp. to remove phenol had appeared slightly difference, because the longtime of acclimation period, where the organisms need a time to adapted for the use of phenol as a sole carbon source . The other reason for this way may be referred to sporulation stage which have a period of time to enter mycelium stage. After the first three days, after the first three days, the increased of phenol degradation by fungi was directly proportional with increase period of incubation. Then, bioremediation efficiency was slightly different. The reveal increased degradation efficiency can be explained by the availability of a carbon source which improves the fungi performances and growth and thereafter, the reduction of carbon source in the solution which is reflected as decrease or an inhibition in the bioremoval process (mortality of the cells) [27].

While the ability of fungi to degrade 1,3 dihydroxy benzene decreased when increased the concentration of it. the results of initial concentration effect of phenol within the range of 10-150 mg L-1. The uptake of phenol increased with the initial concentration up to 120 mg L-1. Then uptake decreased as the initial phenol concentration was increased. The higher uptake at lower concentrations may be due to the presence of more available sites on the adsorbent than the number of phenol ions which are available in solution. The maximum uptake was determined at 120 mg L-1 as 30 mg g-1[36].

Conclusion

The results indicated that a wide range of fungi species have the ability to degrade phenolic compounds. It can be say the bioremoval a wonderful technique for bioremediation of wastewater.

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Table 1. Total counts (TC, calculated per 30 colonies), percentage of fungal counts (%C, calculated per total fungi) and frequency of fungal species (%F, calculated per 10 samples) of various fungal genera and species recovered from 10 samples of wastewater**.**

Genera and species	TC	C%	NCI	F%
Alternaria alternate	3	10.00	1	10
Aspergillus	5	16.67	4	40
A. flavus Link	1	3.33	1	10
A. fumigatus Fresenius	1	3.33	1	10
A. niger Van Teighem	2	6.67	2	20
A. terreus var. *africanus* Fennell and Raper	1	3.33	1	10
Cladosporium cladosporioides (Fres.) de Vries	2	6.67	1	10
Penicillium	4	13.33	2	20
P. aurantiogriseum Dierckx	3	10.00	2	20
P. chrysogenum Thom	1	3.33	1	10
Phoma sp.	1	3.33	1	10
Saccharomyces sp.	14	46.68	2	20
Sterile mycelium	1	3.33	1	10
Total account	30	100.00		

Table 2. Potency of different species of fungi on degradation of phenol derivatives at 0.4% concentrations, incubated at 28º C for 8 days.

Species	Degradation (%) of phenol derivatives at 0.4% concentrations			
	hydroxy-benzene	**2-Naphthol**	**4-Nitrophenol**	**1,3dihydroxy benzene**
Alternaria alternate	85.37*	46.02*	40.23*	84.37*
A. flavus Link	85.57*	33.88*	38.31*	87.08*
A. fumigatus Fresenius	83.11*	37.88*	54.46*	88.02*
A. niger Van Teighem	82.10*	19.33*	37.97*	84.08*
A. terreus var. *africanus*	87.68*	31.07*	42.49*	87.29*
Cladosporium cladosporioides	87.97*	30.58*	48.59*	90.40*
P. aurantiogriseum Dierckx	84.26*	39.26*	38.53*	87.81*
P. chrysogenum Thom	2.2*	31.96*	50.51*	87.23*
Phoma sp.	88.05*	41.37*	43.50*	88.20*
Saccharomyces sp.	83.94*	30.44*	16.05*	86.65*
Sterile mycelium	87.38*	27.14*	54.80*	1.01*

*. The mean difference is significant at the 0.05 level.

Table 3. Potency of different species of fungi on degradation of phenol derivatives at 0.6% concentrations, incubated at 28º C for 8 days.

Species	Degradation (%) of phenol derivaties at 0.6% concentrations			
	hydroxy-benzene	**2-Naphthol**	**4-Nitrophenol**	**1,3dihydroxy benzene**
Alternaria alternate	81.87*	30.61*	37.72*	81.98*
A. flavus Link	83.21*	21.55*	73.70*	87.08*
A. fumigatus Fresenius	82.20*	29.59*	77.36*	84.55*
A. niger Van Teighem	82.21*	44.43*	15.32*	80.52*
A. terreus var. *africanus*	81.87*	29.34*	37.62*	67.80*
Cladosporium cladosporioides	83.51*	50.09*	72.03*	86.47*
P. aurantiogriseum Dierckx	81.69*	34.41*	81.02*	86.29*
P. chrysogenum Thom	26.40*	20.79*	59.38*	85.51*
Phoma sp.	83.19*	30.50*	76.80*	72.59*
Saccharomyces sp.	67.88*	1.1*	50.06*	84.21*
Sterile mycelium	83.86*	38.10*	33.96*	82.55*

*. The mean difference is significant at the 0.05 level.

Table 4. Potency of different species of fungi on degradation of phenol derivatives at 0.8% concentrations, incubated at 28º C for 8 days.

Species	Degradation (%) of phenol derivaties at 0.8% concentrations			
	hydroxy-benzene	**2-Naphthol**	**4-Nitrophenol**	**1,3dihydroxy benzene**
Alternaria alternate	89.27*	35.33*	67.52*	75.82*
A. flavus Link	94.60*	29.90*	51.50*	83.82*
A. fumigatus Fresenius	90.27*	25.43*	57.52*	89.11*
A. niger Van Teighem	66.71*	24.75*	8.05*	65.41*
A. terreus var. *africanus*	79.59*	30.10*	5.58*	59.10*
Cladosporium cladosporioides	95.00*	37.79*	56.64*	89.94*
P. aurantiogriseum Dierckx	93.17*	32.79*	54.87*	91.26*
P. chrysogenum Thom	26.74*	34.14*	45.84*	86.53*
Phoma sp.	81.71*	22.55*	60.00*	24.54*
Saccharomyces sp.	72.88*	32.85*	46.37*	88.42*
Sterile mycelium	93.67*	31.33*	46.90*	1.59*

*. The mean difference is significant at the 0.05 level.

Table 5. Concentrations of photosynthetic pigments of *chlorella* sp. (mg/g fresh wt) under effect of input and output of phenolic compounds degradation at different concentrations by *A.niger* and *P.chrysogenum*.

Phenol sources	Concentrations	Treatments		
		Control	*A.niger*	*P.chrysogenum*
hydroxy-benzene	0.4	4.65±0.14	6.77*±0.23	5.53*±0.10
	0.6	5.00±0.00	5.87*±0.04	7.64*±0.22
	0.8	5.34±0.06	12.05*±.0.15	10.23*±0.07
2-Naphthol	0.4	4.87±0.22	4.64±0.07	4.86±0.15
	0.6	5.94±0.26	7.47*±0.14	6.49*±0.066
	0.8	3.84±0.11	9.14*±0.04	12.83*±0.22
4-Nitrophenol	0.4	3.75±0.08	4.09*±0.04	4.35*±0.08
	0.6	6.84±0.051	5.62*±0.04	6.81±0.12
	0.8	6.25±0.10	6.87*±0.13	8.64*±0.06
1,3dihydroxy benzene	0.4	4.33±0.04	4.56*±0.06	4.65*±0.07
	0.6	4.76±0.13	6.11*±0.03	5.22*±0.08
	0.8	5.9±0.05	4.84*±0.09	12.84*±0.17

409 *. The mean difference is significant at the 0.05 level.